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Appendix 2

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Induction of delayed-type hypersensitivity responses by monoclonal anti-idiotypic antibodies to tumor cells expressing carcinoembryonic antigen and tumor-associated glycoprotein-72

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Abstract. The use of anti-idiotype antibodies as immunogens represents one potential approach to active specific immunotherapy of cancer. Two panels of syngeneic monoclonal anti-idiotypic antibodies were generated. One panel was directed against mAb CC49 and the other to mAb COL-1. mAb CC49 recognizes the pancarcinoma antigen (Ag), tumor-associated glycoprotein-72 (TAG-72), and mAb COL-1 recognizes carcinoembryonic antigen (CEA). Seven anti-idiotypic (AI) antibodies (Ab2) designated AI49-1–7 were generated that recognize the variable region of mAb CC49. These mAb were shown to inhibit the interaction of mAb CC49 (Ab1) with TAG-72 (Ag). Five anti-idiotypic antibodies designated CAI-1–5 were also generated to the anti-CEA mAb, COL-1 (Ab1). These Ab2 were shown to inhibit the interaction between COL-1 (Ab1) and CEA (Ag). Immunization of mice, rats, and rabbits with Ab2 directed against CC49 or COL-1 could not elicit specific Ab3 humoral immune responses, i.e., antibody selectively reactive with their respective target antigens. However, immunization of mice with the CC49 anti-idiotypic antibody (Ab2), designated AI49-3, could induce a delayed-type hypersensitivity response (DTH) specific for tumor cells that express TAG-72. Similarly, immunization of mice with an anti-idiotypic antibody directed against COL-1, designated CAI-1, could induce specific DTH cell-mediated immune responses to murine tumor cells that express human CEA on their surface. These results thus demonstrate that while some anti-idiotype mAb may not be potent immunogens in eliciting Ab3 humoral responses, they are capable of eliciting specific cellular immune responses against human carcinoma-associated antigens. This type of mAb may ultimately be useful in active immunotherapy protocols for human carcinoma.

Key words: Carcinoembryonic antigen – Anti-idiotype antibody – Delayed-type hypersensitivity

Introduction

Among the more studied human carcinoma-associated antigens are carcinoembryonic antigen (CEA) [14] and tumor-associated glycoprotein-72 (TAG-72) [5]. CEA is a 180-kDa glycoprotein expressed on the majority of colon, rectal, stomach, and pancreatic tumors [38], and 50% of breast carcinomas [48] and 70% of lung adenocarcinomas [53]. CEA is also expressed on normal colonic epithelium. TAG-72, initially defined by monoclonal antibody B72.3, is a 10^6-kDa mucin found on the cell surface of colorectal, gastric, pancreatic, ovarian, endometrial, mammary, and non-small-cell lung carcinomas [5]. TAG-72 is not appreciably expressed on a range of normal human tissues with the exception of normal secretory endometrium [49] and transitional mucosa, the mucosa adjacent to the tumor mass [55]. Monoclonal antibodies to TAG-72 [6, 10, 32] and to CEA [2, 15, 45] have both had success in radiolocalization of tumors in patients. Both of these antigens represent potential targets of active specific immunotherapy.

The use of anti-idiotypic antibodies as an immunogen represents one potential approach of active specific immunotherapy. The internal-image anti-idiotypic antibody (Ab2) that recognizes the paratope or the idiotype (Ab1) may mimic the antigenic determinant recognized by the idiotype. Since these complementary idiotypic and anti-idiotypic interactions may function to regulate immune responses [20], an Ab2 can potentially be utilized as a surrogate immunogen to induce specific immune responses [40]. These idiotype/anti-idiotype network systems have also been shown to play a role in the regulation of T cell immunity via immunoglobulin and T cell receptor idiotypes [9, 41, 42].

Some of the studies described in this paper were in partial fulfillment of requirements for the completion of Dr. Irvine's dissertation at the George Washington University

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Traditionally, most investigators characterize the internal image of anti-idiotypic antibodies by the following criteria: (a) Ab2 must be able to bind to the Ab1 idiotype, (b) Ab2 must inhibit Ab1 binding to antigen and (c) Ab2 must be able to induce an anti-anti-idiotype or "Ab3" immune response reactive with the antigen, which could be either a cell-mediated or humoral immune response. Recently, the use of anti-idiotypic antibodies as a vaccine has been reported to produce protective immunity against parasites, bacteria, and viral infections [16, 34, 43]. This strategy is also being pursued in tumor antigen systems for carcinoma [17, 18, 29, 52], melanoma [22, 28] and sarcoma [7] in both rodent [7, 42] and human [17, 22, 28, 29] systems.

We present data here on the generation and characterization of two panels of monoclonal anti-idiotypic antibodies that recognize the variable regions of mAb CC49 and COL-1, that react with TAG-72 and CEA, respectively. CC49 and COL-1 mAb were selected because of their defined pattern of reactivity specific for certain types of human tumors versus the vast majority of normal tissues [38]. COL-1 mAb has also been shown to react specifically with CEA but not with the closely related molecule nonspecific cross-reacting antigen that is found on the surface of human granulocytes. COL-1 has a K_a of 1.36×10^9 M^{-1} and has been shown to react with a protein epitope on CEA [26]. mAb CC49 has a K_a of 1.2×10^9 M^{-1} and has been shown to react with a carbohydrate epitope [27]. In ongoing clinical trials, radiolabeled forms of both mAb CC49 and COL-1 have been shown to localize carcinoma selectively in colon cancer patients (S. Larson, and B. Yu, personal communication).

We report here that both sets of anti-idiotypic antibodies could specifically inhibit Ab1 binding to their antigen. However, when the Ab2 were used as an immunogen, an Ab3 humoral immune response of antibodies reactive with the original antigen could not be detected. We do demonstrate, however, that one of the anti-idiotypic antibodies that recognizes the anti-TAG-72 mAb CC49, could induce delayed-type hypersensitivity (DTH) responses specific for tumor cells that express TAG-72. One COL-1 anti-idiotypic antibody, CAI-1, could similarly elicit DTH responses to CEA on the surface of murine tumor cells.

Materials and methods

Animals. Balb/c and C57BL/6 female mice between 6 and 12 weeks old, Wistar rats and New Zealand rabbits were obtained from the Frederick Cancer Research Facility (FCRF), quarantined and maintained in the Health Center Animal Resources Facility at NIH. Female athymic mice (*nu/nu*) with Balb/c background, also obtained from the FCRF, were used to induce hybridoma ascites in this study.

Cells. The LS-174T colon carcinoma cell line [50], obtained from the American Type Culture Collection (ATCC, Rockville, Md.) was grown as described previously [39]. The MRC-5 human embryonic fibroblast cell line was acquired from the ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS). The MC-38 murine colon adenocarcinoma cell line was a gift from Dr. S. Rosenberg [11]. The MC-38 line transduced with the CEA gene (MC-38-CEA-2) was obtained from Dr. P. Robbins [44]. Both lines were maintained in DMEM containing 10% FCS. The OVCAR-3 human ovarian carcinoma cell line obtained from Dr. David Segal was maintained in ascites of nude mice on a Balb/c background. The tumor cells were harvested from ascites and grown in RPMI-1640 complete medium supplemented with 2 mM glutamine, 1 mM sodium pyruvate, fungizone (0.25 μg/ml), streptomycin (50 μg/ml) and 15% heat-inactivated FCS. All murine hybridoma cell lines were passaged in RPMI-1640 complete medium as described above. Cells were cultured at 37° C in a humidified incubator containing 7.0% CO_2. Monolayers were detached from culture flasks with 0.1% trypsin containing 0.5 mM EDTA.

Monoclonal antibodies. Two panels of mouse mAb recognizing the two distinct human tumor-associated antigens, TAG-72 and CEA, were used. One panel of anti-TAG-72 mAb (B72.3, CC11, 15, 29, 30, 40, 46, 49, 83, 92, and 112) recognized multiple epitopes of the TAG-72 molecule [27]. The panel of anti-CEA mAb (COL-1, 4, 6, 7, and 11), was generated as previously described, and recognized different epitopes of CEA [26]. D612 mAb was utilized as an isotype-matched control for these studies. D612 has been reported to react with human gastrointestinal carcinoma and to normal gastrointestinal tissue. It is non-reactive with CEA [39]. A rat monoclonal anti-idiotypic antibody specific for mAb B72.3, designated AI72.3, was utilized as a control for the CC49 Ab2 fine-specificity studies.

Production of anti-idiotypic mAb species (Ab2) to CC49 (Ab1) and COL-1 (Ab1). Balb/c mice were immunized by intraperitoneal (i.p.) and subcutaneous (s.c.) injections of 50 μg/200 μl of either CC49 or COL-1 purified mAb coupled to keyhole limpet hemocyanin (KLH) (Sigma, St. Louis, Mo.) emulsified in complete Freund's adjuvant (Sigma, St. Louis, Mo.) as previously described [42]. Animals were then boosted weekly with the immunogen emulsified in incomplete Freund's adjuvant. The initial boost contained 50 μg/200 μl whereas subsequent boosts contained 20 μg. Prior to fusion, the mice were given a final intravenous (i.v.) boost of 10 μg CC49/KLH or COL-1/KLH conjugates diluted in 100 μl phosphate-buffered saline (PBS). The fusion was performed 3 days later according to the standard methods for hybridoma technology [19]. Briefly, the splenic lymphocytes derived from the mice immunized with either CC49 or COL-1 mAb were harvested and mechanically dispersed over a wire mesh screen (Fenco Cage Products, Boston, Mass.). Subsequently, these cells were fused with the non-immunoglobulin-secreting mouse myeloma cell line P3-NS1 Ag4 ([23], ATCC no. TIB-18) using a 50% solution of polyethylene glycol 1500 (BDH Chemicals Ltd., Poole, England) and cultured in hypoxanthine/aminopterin/thymidine selection medium as previously described [38].

Screening of anti-idiotype hybridoma supernatants. Initial screening of the CC49 Ab2 hybridoma supernatants was done by a solid-phase enzyme-linked immunosorbent assay (ELISA) using a modification of an indirect method for the detection of bound immunoglobulin [42]. A sample containing 50 ng purified CC49 $F(ab')_2$ or a purified preparation of murine polyclonal IgG $F(ab')_2$ fragments diluted in PBS was coated to each well of 96-well polyvinyl chloride flat-bottom microtiter plates (Dynatech, Chantilly, Va.) and incubated overnight at 4° C. For every immunoassay described in this section, antigen-coated microtiter wells were treated with 100 μl 3% bovine serum albumin (BSA, Sigma, St. Louis, Mo.) in PBS for 1 h at 37° C to prevent the non-specific binding of antibody to the plates. A 50-μl sample of either a 1:3 dilution of hybridoma tissue-culture supernatants or various dilutions of purified Ab2 mAbs was then added to each well. Following a 1-h incubation, 50 μl horseradish-peroxidase-conjugated rabbit anti-(mouse IgG Fc) serum (Jackson Immunoresearch Laboratories, West Grove, Pa.) was added (1:2500) to each well for 1 h, 37° C. After a wash step, any remaining bound immunoglobulin was revealed by a 15-min incubation with 100 μl substrate solution containing 0.01% H_2O_2 and 2.8 mM *o*-phenylenediamine dihydrochloride substrate (Sigma, St. Louis, Mo.) diluted in 0.1 M phosphate/citrate buffer, pH 5.0. The reactions were stopped by addition of 25 μl 4 M H_2SO_4. Plates were read on a Bio-tek microplate ELISA reader EL310 (Winooski, VT) at an absorbance of 490 nm. Those hybridoma supernatants that contained mAb that specifically recognized

CC49 F(ab')2 fragments but not murine I (ab')2 fragments were selected for further characterization of purified an i-CC49 mAb.

The COL-1 Ab2 hybridoma supern tants were screened using an inhibition solid-phase radioimmunoassa / (SPRIA) where the supernatants were tested for the presence of imu unoglobulin that could inhibit ^{125}I-radiolabeled CEA (International En: yme, San Diego, Calif.) from binding to COL-1 mAb (Ab1) but not inh bit binding to an isotype-identical anti-CEA mAb, COL-4 (IgG2a). In this assay, COL-1 and COL-4 mAb were coated overnight to each w ll of round-bottom microtiter plates (100 ng/50 µl) at 4° C. The plates v ere blocked from non-specific protein binding with 100 µl 5% bovine s rum albumin (BSA) diluted in PBS for 1 h at 37° C. Samples containing 50 µl hybridoma supernatants at a 1:2 dilution were incubated for 1 h at 37° C. Following the wash step, which removed unbound immu oglobulin, ^{125}I-labeled CEA (50 000 cpm/25 µl) was added to each w ll for 1 h at 37° C. The plates were washed and exposed overnight at −7 l° C to Kodak XAR film with a lightning-plus screen (Dupont, Wilmingt n, Del.). Idiotype-specific supernatants were selected on the basis of th ir ability to inhibit the labeled CEA from binding to COL-1 mAb but nc to COL-4 mAb.

Selection of the CC49 Ab2 that inhibit Ag Ab1 interaction. An inhibition assay was developed to characterize th anti-idiotypic antibodies to CC49 mAb. Purified TAG-72 (0.349 mi 750 µl) was dried down overnight at 37° C to each well of round-bott n microtiter plates (Dynatech, Chantilly, Va.). TAG-72 was purified as reviously described from LS-174T colon carcinoma xenografts [46]. C ne unit of purified TAG-72 is defined as the amount of TAG-72 found i n one microgram of a standard tumor extract expressing TAG-72 [21]. n separate microtiter reaction plates that contain no antigen, 10 ng pur fied CC49 protein (50 µl) was coincubated with 50 µl of either dilution s of Ab2 tissue-culture supernatants or various concentrations of purif ed immunoglobulin for 1 h at 37° C. Next, 50 µl mixture was transfe red to the TAG-72 detection plates and incubated for 1 h at 37° C. B und CC49 mAb was detected with ^{125}I-radiolabeled goat anti-(mouse Ig G H+L chain) specific antisera (75 000 cpm/25 µl) (Becton-Dickinson, S n Jose, Calif.). The percentage inhibition was calculated by the followin formula:

$$100 - \left\{ \frac{100 \times [\text{test sample } ^{125}\text{I (cpm)} - \text{background } ^{125}\text{I (cpm)}]}{\text{total } ^{125}\text{I (cpm)} - \text{background } ^{125}\text{I (cpm)}} \right\}$$

The hybridoma cells that secreted mAb t at interfered with CC49 mAb (Ab1) binding to purified TAG-72 were cloned twice and injected into mice for ascites production.

Isotyping of monoclonal anti-idiotypic antibodies. A SPRIA was performed as described previously for the isotype determination of these Ab2 [38].

Purification of anti-idiotypic monoclonal antibodies (Ab2). For the purification of the anti-idiotypic antibodies o CC49, the immunoglobulin was precipitated from the ascites fluid w ith 40% saturated ammonium sulfate at 4° C for 3 h. The immunoglobu in was then dialyzed overnight against 20 mM TRIS/HCl (pH 7.0) at 1 applied to an ion-exchange column (SAX protein DEAE; Waters, Division of Millipore, Marlborough, Mass.) by high-performance liq id chromatography. Antibody was eluted with a salt gradient ranging f om 0 to 0.5 M NaCl diluted in 20 mM TRIS/HCl (pH 7.0). Fractions co taining the anti-idiotype mAb were analyzed by sodium dodecyl sulfa e/polyacrylamide gel electrophoresis followed by Coomassie blue st ining to reveal protein bands. Each fraction was assayed for reactivit by the indirect anti-idiotype binding assay to CC49 F(ab')2. The fra tions containing anti-idiotype mAb were pooled and dialyzed extensi ely against PBS. The protein concentration was determined by the met od of Lowry et al. [31].

The anti-idiotypic antibodies reactive with COL-1 were purified over a column containing *Staphylococcus aur us* protein-A-Sepharose CL4B (SPA-Sepharose) beads (Pharmacia, Up sala, N.Y.). A 1.5-g sample of dry SPA-Sepharose beads was swollen i 0.1 M $NaPO_4$ buffer, pH 8.0, for 30 min and 1–2 ml murine hybridom a ascites fluid was added to the beads and rotated at room temperature f r 30 min. Following extensive washing with the 0.1 M $NaPO_4$ buffer, H 8.0, bound immunoglobulin was eluted with 0.1 M sodium citrate buf er pH 3.0–4.5. Purified immunoglobulin was immediately neutralized with 1 M TRIS and dialyzed against PBS. The fractions were characterized and pooled as described above.

Radiolabeling of monoclonal antibodies with iodine-125. The mAb COL-1, CC49, AI49-1–7, CAI-3, and CEA were labeled with sodium iodide ($Na^{125}I$) using a modification of the Iodogen technique [12]. Iodogen (Pierce Chemical, Rockford, Ill.) was diluted in chloroform to 10 mg/ml and 20-ml aliquots were evaporated under a stream of nitrogen and stored at −20° C until use. A 50-µg aliquot of antibody or 200 µg antigen diluted in PBS and 0.5 mCi $Na^{125}I$ were added to the iodogen tubes. After a 2-min incubation at room temperature, the protein was removed from the insoluble iodogen and the unincorporated ^{125}I was separated from the antibody by gel filtration through Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, N.J.).

Antibody coupling with KLH. Monoclonal antibodies were coupled to the carrier protein keyhole limpet hemocyanin (KLH, Sigma Chemical, St. Louis, Mo.) by chemical cross-linking in the presence of glutaraldehyde (Sigma Chemical, St. Louis, Mo.) as described [33].

Ab2 induction of the Ab3 humoral response. Balb/c mice, Wistar rats and New Zealand white rabbits were immunized with anti-idiotypic antibodies AI49-1–6 to examine the specificity of the humoral Ab3 immune response within and across species boundaries. Wistar rats were immunized with mAb AI49-3, 4 and 5. Three animals per group of mice and rats were immunized subcutaneously with either 25 µg or 50 µg purified anti-idiotypic antibodies coupled to KLH and emulsified in complete Freund's adjuvant. Subcutaneous boosts of the same amount of immunogen emulsified in incomplete Freund's adjuvant were administered every 2 weeks. Rabbits were immunized subcutaneously at multiple sites with 50 µg anti-idiotypic antibodies coupled to KLH, also emulsified in adjuvant as described above. All animals were bled every 2 weeks either 7 days following each boost for mice and rats or just prior to each boost for the rabbits. Two strains of mice (Balb/c and C57BL/6) and New Zealand white rabbits were also immunized as described above with three of the anti-idiotypic mAb to CEA designated CAI-1, CAI-3 and CAI-5.

Quantification of TAG-72-reactive antibody. Serum samples from mice and rats were collected from the tail vein into Natelson heparanized collecting tubes (Government Municuns Services, Washington, D.C.). All serum samples were tested in a modified SPRIA for antibody reactivity to purified TAG-72. TAG-72 (0.174 U/50 µl) and BSA (100 ng/50 µl) diluted in PBS were each dried down to microtiter wells overnight at 37° C. Serial dilutions of serum starting at 1:100 were incubated with the antigen on the plate for 1 h at 37° C. For the detection of bound mouse immunoglobulin, ^{125}I-labeled goat anti-(mouse IgG H+L chain) specific antisera (75 000 cpm/25 µl) was incubated for 1 h at 37° C. Bound rat immunoglobulin was determined utilizing a rabbit anti-(rat IgG H+L chain) specific linker (1:2500) (Jackson Immunoresearch Laboratories, West Grove, Pa.) followed by ^{125}I-labeled *S. aureus* protein A (SPA) (50 000 cpm/25 µl). Rabbit antiserum binding to TAG-72 or BSA was detected by ^{125}I-labeled SPA (50 000 cpm/25 µl).

Quantification of antibodies reactive with CEA. Samples of mouse and rabbit sera were quantified for anti-CEA antibodies by ELISA. Microtiter plates were dried down overnight at 37° C with 100 ng/well purified CEA. The wells were incubated with dilutions of mouse or rabbit antiserum, preimmunization serum, or the anti-CEA mAb, COL-1. Bound antibody was detected with horseradish-peroxidase-conjugated goat anti-(mouse IgG) antiserum or similarly conjugated donkey anti-(rabbit IgG) antiserum (Becton Dickinson, San Jose, Calif.). The complex was detected using the *o*-phenylenediamine chromogen as described above.

Ab2 induction of antigen-specific cell-mediated hypersensitivity responses. The induction of cell-mediated immunity to TAG-72 by immunization with anti-idiotypic mAb AI49-1–7 was explored using a delayed-type hypersensitivity assay [23]. Balb/c mice were immunized twice at 2-week intervals with 1.5×10^6 irradiated (40 Gy) hybridoma

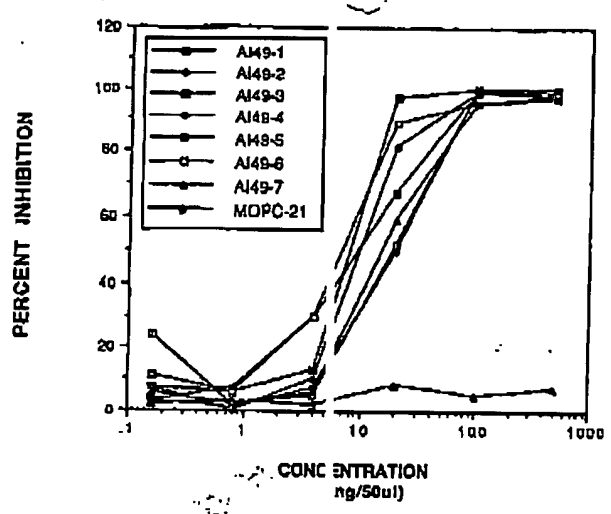

Fig. 1. Inhibition of CC49 mAb (AI I) binding to TAG-72 by Ab2 species. Purified TAG-72 (0.35 U/50 µ 'well) was dried down overnight, 37°C, to microtiter plates. In a separa e reaction plate with no antigen bound, purified CC49 mAb (20 ng/50 I) was coincubated with fivefold dilutions of anti-idiotypic mAb AI49- −7, or control IgG, MOPC-21, starting at 1000 ng/50 µl for 1 h, 37°C Samples containing 50 µl mixture were then incubated on the TAG-' 2 detection plate for 1 h. Bound CC49 mAb was detected by a subsequ it 1-h incubation with ^{125}I-radiolabeled goat anti-(mouse IgG) (heavy- nd light-chain-specific) antisera (75 000 cpm/25 µl). The percentage i hibition was calculated as described in Materials and methods

cells secreting the anti-idiotypic antibo y, AI49-3, or human tumor cells that express TAG-72, emulsified in inc mplete Freund's adjuvant (IFA). X-irradiated hybridoma cells secreting a control isotype-matched mAb (COL-12) emulsified in IFA, PBS em ulsified in IFA, and PBS alone were utilized as control immunogens. n a separate experiment, Balb/c mice were immunized with 100 µg p rified anti-idiotypic antibodies AI49-3−5 coupled to KLH in the man ner described above. Mice were challenged with an injection in one ootpad of 5×10^5 X-irradiated human ovarian carcinoma cells (OVC AR-3), which express TAG-72, 7 days following the final boost. As a control for a non-specific DTH response, each mouse received an inject on of 5×10^5 X-irradiated MRC-5 human fibroblast cells in the opposite footpad and expressed in "mil" (0.0254 mm). After 48 h, footpad thickness was measured with a micrometer. DTH was calculated as the differ nce of footpad swelling between hind footpads. This experiment was rep ated four times with three to five mice per group and readings were made in a blind manner.

C57BL/6 mice were also utilized t analyze the specific DTH responses induced by the purified COL-1 nti-idiotypic antibodies. In three separate experiments, four to six mice per group were immunized intraperitoneally with X-irradiated hybr doma cells (40 Gy) secreting COL-1 Ab2, CAI-1, or X-irradiated hyl ridoma cells secreting a control immunoglobulin D612. Seven days fo lowing the last immunization, 5×10^5 X-irradiated (200 Gy) human CEA-transduced murine tumor cells, MC-38-CEA-2, in 20 µl PBS wer injected into one hind footpad and 20 µl PBS containing 5×10^5 X-irr diated cells from the non-transduced cell line, MC-38, were injected into the other hind footpad. The thickness of the footpads was measure 48 h later as described. The P values were determined utilizing Studer 's t-test of significance [47].

Results

Generation of anti-idiotypic antibodies to mAb CC49

Spleens from mice immunized with CC49 mAb coupled to KLH were subsequently harvested for hybridoma production. The supernatants from a total of 2750 wells were screened in a solid-phase ELISA for the presence of antibodies that bound to CC49 F(ab')$_2$ fragments versus control murine polyclonal F(ab')$_2$. Out of 2750 wells screened, 26 were reactive with CC49 F(ab')$_2$ but not with the control murine F(ab')$_2$ fragments. The remaining wells were negative to both CC49 and control F(ab')$_2$. No mAb were generated that recognize both CC49 and the control IgG F(ab')$_2$.

In order to determine whether these hybridoma supernatants contain immunoglobulin reactive with sites associated with the paratope of mAb CC49, the CC49-reactive supernatants were screened in a competition radioimmunoassay. Out of 26 anti-idiotypic antibody supernatants, 10 inhibited mAb CC49 from binding to TAG-72. These Ab2 represent the portion of the total population that could be classified as potential Ab2 that may bear the image of an epitope on TAG-72. Seven of the wells containing anti-idiotypic antibodies to mAb CC49 that most efficiently inhibited CC49 binding to TAG-72 were selected for ascites production and further characterization. These mAb were designated AI49 (anti-idiotypes to CC49) 1–7.

Binding reactivity of purified anti-idiotypic antibody, AI49-1−7. Studies were undertaken to determine if the purified Ab2 species, AI49-1–7, could inhibit mAb CC49 from binding to the TAG-72 antigen. As seen in the radioimmunoassay results shown in Fig. 1, all of the purified Ab2 specifically inhibited mAb CC49 (Ab1) from binding to purified TAG-72 while the irrelevant control antibody, MOPC-21, failed to inhibit binding. These results suggested that the purified anti-idiotypic antibodies recognize site(s) proximal to the CC49 antigen combining site.

Specificity of monoclonal anti-idiotypic antibodies (Ab2) for a site unique to mAb CC49 (Ab1). In a previous report [27], we demonstrated that many of the anti-TAG-72 "CC" mAb, including CC49, were shown to cross-compete with each other in a reciprocal competition radioimmunoassay. A radioimmunoassay was designed to determine if the Ab2 species AI49-1−7 recognize determinants common to some or all of a panel of ten anti-TAG-72 mAb, or only recognize a determinant unique to the mAb CC49. AI49-1−7 were radiolabeled and tested in direct-binding radioimmunoassay for immunoreactivity to a panel of CC mAb. As shown in Table 1, the ^{125}I-radiolabeled AI49-1–7 mAb species bound uniquely to the mAb CC49 idiotype but not to any of the other isotypes on the anti-TAG-72 mAb species or the irrelevant control mAb, COL-3. As a positive control, ^{125}I-labeled goat anti-(mouse IgG) antiserum was shown to bind to all wells containing IgG. Therefore, mAb AI49-1–7 recognize epitopes restricted to CC49 among the panel of CC mAb.

Table 1. Binding reactivity of anti-idiotyp c antibodies (Ab2) AI49-1–7 to a panel of anti-TAG-72 mAb[a]

mAb (Ab1)	Isotype	^{125}I- abeled AI49 mAb (Ab2) (cpm)							
		AI4 -1	AI49-2	AI49-3	AI49-4	AI49-5	AI49-6	AI49-7	GAM
B72.3	IgG1	6.	79	63	79	19	45	109	4276
CC11	IgG1	6·	36	104	45	24	6	54	5430
CC15	IgG2b	7:	42	54	35	153	94	194	4859
CC29	IgG1	8!	0	69	54	37	5	95	4174
CC40	IgG1	7!	35	63	99	63	4	105	3238
CC46	IgG1	7!	54	68	68	40	169	155	4851
CC49	IgG1	310	12056	9110	6938	10072	5597	3498	2475
CC50	IgG1	16	47	130	78	4	35	82	5348
CC83	IgG1	17:	89	64	184	79	112	34	4304
CC112	IgM	13:	57	74	75	39	1	147	283
COL-3	IgG1	10·	57	49	55	47	4	89	3569
PBS	NA[b]	17	25	19	95	31	28	53	24

[a] Microtiter plates were coated overnight t 4° C with 50 ng/50 µl differ- ent mAb. Following an incubation with ph sphate-buffered saline (PBS) containing 5% bovine serum albumin to bl ck non-specific protein bind- ing, ^{125}I-radiolabeled AI49 anti-idiotypic . ntibodies or ^{125}I-radiolabeled goat anti-(mouse IgG) (GAM) (50000 cpm/well) were incubated on the coated microtiter plates for 1 h. Plates were washed and bound immuno- globulin was determined as cpm bound

[b] NA, not applicable

Table 2. Fine binding specificity of an -idiotypic antibodies (Ab2), AI49-1–7, for the CC49 idiotype (Ab1)[a]

Inhibitor Ab2 mAb	Inhibition 50% (ng)[b]					
	AI49-3	Index-3[c]	AI49-5	Index-5	AI49-6	Index-6
AI49-1	2.4	3.8	2.5	0.9	5.0	7.8
AI49-2	7.8	12.2	2.7	1.0	6.5	10.2
AI49-3	0.6	1.0	<0.6	<0.2	1.5	2.3
AI49-4	105.0	164.1	5.0	1.9	12.5	19.5
AI49-5	16.0	25.0	2.7	1.0	7.0	11.0
AI49-6	9.0	14.1	<0.6	<0.2	0.6	1.0
AI49-7	5.5	8.6	<0.6	<0.2	3.8	5.9
AI72.3	2000.0	3125.0	2000.0	>741.0	2000.0	3125.0

[a] Microtiter wells coated with CC49 F ab')$_2$ fragment (50 ng/50 µl) were incubated with fivefold dilutions of different Ab2 or the irrelevant control anti-idiotypic antibody to B72.3, AI72.3, for 1 h at 37° C. Sub- sequently, ^{125}I-radiolabeled AI49 Ab2 mA , AI49-3, AI49-5 and AI49-6, were added to the antigen plate (75000 cp m/25 µl) and the mixture was incubated overnight, 4° C. The percentag inhibition was calculated as described

[b] Quantity (ng) required to inhibit ^{125}I- adiolabeled Ab2 binding by 50% (I_{50})

[c] Index the experimental competitor I_{50} alues divided by the I_{50} value obtained by an Ab2 competing with itself

Fine specificity of the AI49 mAb (Ab2) binding to the CC49 idiotype (Ab1). Reciprocal antibody competition radioimmunoassays were designed to "map" the binding location of the different Ab2 species, AI49-1–7, to the idiotype of mAb CC49 and to delineate whether fine binding differences exist between them. Table 2 summarizes the data from these mapping studies. The panel of Ab2 was analyzed for their ability to inhibit radiolabeled Ab2 from binding to mAb CC49 (Ab1); AI49-3, 5, and 6 were radiolabeled for this study. For these studies, complete inhibition curves were generated for each competitor Ab2 and the quantity (ng) required to inhibit the radiolabeled Ab2 by 50% (I_{50}) was determined. From these values, an index was derived by dividing the experimental competitor I_{50} values by the I_{50} value obtained by an Ab2 competing with itself. The relatively low indices indicate that all of the Ab2 mAb could efficiently inhibit the labeled anti-idiotypic antibodies from binding to mAb CC49 (Ab1) (Table 2). A control anti-idiotypic antibody AI72.3, which does not react with CC49, failed to compete for binding.

Fine differences in binding were noted between the Ab2 species (Table 2). AI49-4 required 164-fold more antibody for 50% inhibition than the homologous competitor AI49-3, indicating that it may recognize a related but different epitope on mAb CC49 or may have a lower affinity than that of AI49-3. AI49-5 appeared to have a lower affinity to mAb CC49 compared to the other types of Ab2. In some cases (AI49-3, 6, 7), less competition antibody was required to cause 50% inhibition than when AI49-5 was used as a competitor against itself. mAb AI49-2, 4, 5 appear to recognize related but different epitopes as AI49-6.

Generation of monoclonal anti-idiotypic antibodies to carcinoembryonic antigen

Spleens from mice immunized with COL-1 mAb coupled to KLH were harvested for hybridoma production. Hybridoma supernatants from 5000 wells were screened in a solid-phase competition radioimmunoassay for the presence of antibody that could inhibit ^{125}I-radiolabeled CEA from binding to either mAb COL-1 or its isotype-identical mAb COL-4. Five supernatants out of 5000 wells screened (0.1%) contained immunoglobulin that specifically inhibited radiolabeled CEA binding to COL-1 but not to mAb COL-4. These five anti-idiotypic antibodies were designated the COL-1 anti-idiotypic antibodies, CAI-1–5. No supernatants were observed that could inhibit CEA from binding to mAb COL-4.

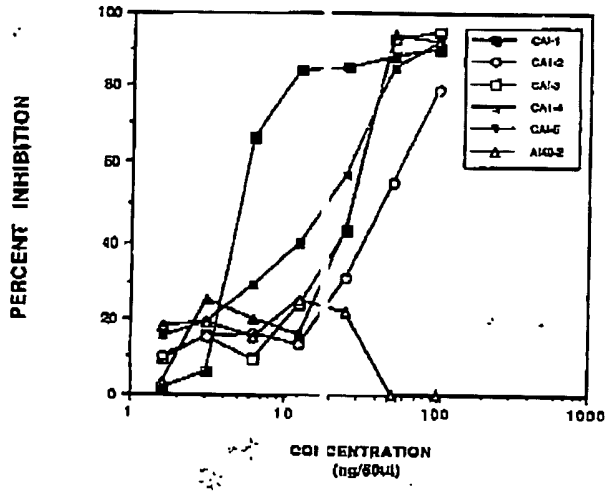

Fig. 2. Inhibition of COL-1 (Ab1) bi ding to carcinoembryonic antigen (CEA) (Ag) by Ab2. Purified CEA (5 ng/well) was dried down overnight to each well on microtiter plates In a separate blank reaction plate, twofold dilutions of the purified anti idiotypic antibodies, CAI-1 (■), CAI-2 (○), CAI-3 (□), CAI-4 (▲), (AI-5 (⊞) as well as control mAb, AI49-2 (△) (starting at 100 ng/25 µl) were coincubated with ^{125}I-radiolabeled COL-1 mAb (25 000 cpm/25 µ l) for 1 h, 37° C. Samples containing 50 µl mixtures were then incuba ed for 1 h on the CEA detection plates. The radioactivity (cpm) was (etected and percentage inhibition was determined as described in Mater als and methods

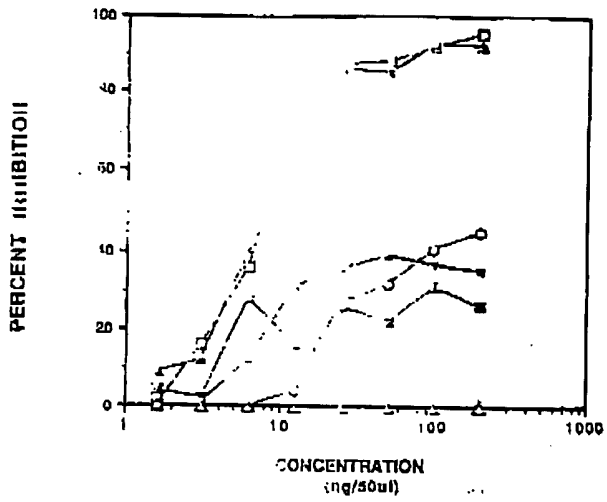

Fig. 3. Fine specificity of the anti-idiotypic antibodies (Ab2), CAI-1–5 binding to the COL-1 (Ab1) idiotype. Microtiter wells were coated overnight, 4° C, with 50 ng/50 µl purified COL-1 mAb (Ab1). Following a step to block non-specific protein binding, the plates were incubated for 1 h with twofold dilutions of purified anti-idiotypic antibodies, CAI-1 (■), CAI-2 (○), CAI-3 (□), CAI-4 (▲), CAI-5 (⊞) as well as a control immunoglobulin, AI49-2 (△) at a starting concentration of 200 ng/50 µl. Subsequently, ^{125}I-radiolabeled anti-idiotypic mAb, designated CAI-3 (50 000 cpm/25 µl), was added to the plates and incubated overnight at 4° C. The percentage inhibition was calculated as described in Materials and methods

Table 3. Binding specificity of the C)L-1 anti-idiotypic antibodies to a panel of anti-(carcinoembryonic anti; en) (anti-CEA) monoclonal antibodies[a]

Ab2 Inhibitor TCS	Isotype	Anti-CEA mAl s (Ab1) (% inhibition)				
		COL-1	COI -4	COL-6	COL-7	COL-11
CAI-1	IgG2a	100	10	8	0	17
CAI-2	IgG1	96	1	7	16	23
CAI-3	IgG2a	98	0	8	16	0
CAI-4	IgG2b	99	3	0	0	0
CAI-5	IgG2b	92	5	0	0	0
NS-1	NA[b]	0	0	0	0	0

[a] Microtiter plates were coated with a panel of five anti-CEA monoclonal antibodies (100 ng/50 µl) and each was incubated with Ab2 hybridoma tissue-culture supernatants (TCS) or control TCS from NS-1 myeloma cells for 1 h. 37° C. The pla es were washed and incubated for 1 h with ^{125}I-radiolabeled CEA (50 00 · cpm/25 µl). The percentage inhibition of the radiolabeled CEA bin ng to the anti-CEA monoclonal antibodies was calculated as describ in Materials and methods

[b] Not applicable

Binding reactivity of purifie anti-idiotypic antibodies, CAI-1–5. Studies were unde aken to analyze and compare the binding reactivities c the purified Ab2 species, CAI-1–5. In the competition r idioimmunoassay shown in Fig. 2, CAI-1–5, specifically inhibited ^{125}I-radiolabeled mAb COL-1 (Ab1) from bindi ng to purified CEA (Ag). A control mAb directed at the CC 49 idiotype failed to inhibit the labeled mAb COL-1 bind ng. The five different Ab2 demonstrated inhibition curv es that had three distinct slopes suggesting that these A b2 may recognize different sites on the COL-1 idiotype (Ab1). Ab2 mAb, CAI-1, inhibited COL-1 binding with the highest relative affinity; the mAb required only a.. ng to achieve 50% inhibition. Ab2 mAb CAI-3 and CAI-4 demonstrated superimposable curves suggesting that they may recognize very related or identical epitopes. CAI-2 and CAI-5 have similar slopes suggesting that they react with similar epitopes; CAI-5 (Ab2) appeared to have a higher affinity than the rest of the Ab2 CAI-2–4 to mAb COL-1 (Ab1).

Anti-idiotypic monoclonal antibodies, CAI-1–5, recognize a site unique to COL-1. Previous studies using reciprocal competition RIA have shown various degrees of cross-reactivity among the anti-CEA COL mAb series. Specifically, COL-1, 4, 6, 7, and 11 all cross-compete with each other for CEA binding and cannot be distinguished from each other on the basis of these assays [26]. A competition radioimmunoassay was designed to determine if the COL-1 Ab2 mAb recognize determinants found on a panel of anti-CEA mAb. As shown in Table 3, all of the Ab2 inhibited the binding of [illegible] to mAb COL-1 species but not the binding of ^{125}I-radiolabeled CEA to any of the other anti-CEA mAb. These data suggested that these Ab2 specifically recognize epitopes unique to the COL-1 idiotype.

Fine binding specificity of the anti-idiotypic antibodies CAI-1–5 for the COL-1 idiotype. A antibody competition radioimmunoassay was designed to map the binding of the different Ab2 mAb to the idiotype of the mAb COL-1

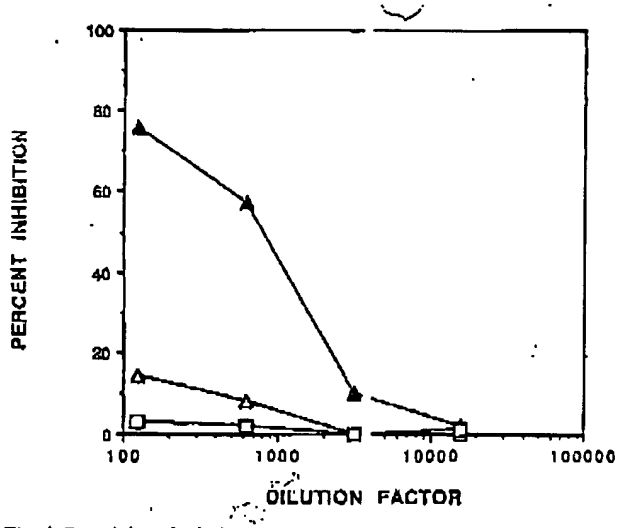

Fig. 4. Reactivity of rabbit serum (Ab3) b nding to the CC49 anti-idiotypic antibody (Ab2) immunogen, AI49-5. One rabbit was immunized twice at 2-week intervals with the CC49 A b2, AI49-5. Fivefold dilutions of preimmunization (Δ), rabbit anti-AI49-5 serum (▲) and a control hyperimmune serum against another Ab2, AI49-3 (□) (starting at 1:125) were tested for reactivity to purified AI49-5 Ab2. Rabbit anti-(mouse Ig) Ab3 serum was diluted in phosphate-buffered saline (PBS) with 1% mouse serum to absorb out anti-F c rabbit responses. Microtiter plates coated with purified AI49-5 mAb (100 ng/well) were incubated with different dilutions of rabbit antibodies for 1 h, 37° C. ^{125}I-radiolabeled CC49 mAb (50 000 cpm/25 μl) was sequentially added to the plates and incubated for 1 h at 37° C. The percentage inhibition of CC49 mAb binding was calculated as described in Materials and methods

(Ab1) to delineate whether there are fine binding differences between the Ab2 species. Figure 3 illustrates the results of a competition assay in which the panel of Ab2 were analyzed for their ability to inhibit ^{125}I-labeled CAI-3 Ab2 mAb from binding to the COL-1 idiotype (Ab1). As shown, mAb CAI-4 (Ab2) completely inhibited labeled CAI-3 Ab2 binding to COL-1 Ab1, producing a curve superimposable onto the CAI-3 Ab2 inhibition curve of its own binding to COL-1 (Ab1). These data suggested that CAI-3 (Ab2) recognizes the same site as CAI-4 (Ab2) on COL-1 (Ab1). The remaining Ab2 species could only partially inhibit mAb CAI-3 binding, indicating that the Ab2 differ in affinity or that they recognize distinct epitopes on the COL-1 idiotype. The irrelevant control antibody failed to inhibit ^{125}I-CAI-3 binding to mAb COL-1. These results suggest that the CAI Ab2 antibodies can be distinguished by their binding to COL-1.

Analysis of Ab3 humoral immune responses induced by Ab2

Humoral Ab3 immune responses elicited by AI49-1–6. The anti-idiotypic antibodies to CC49 (i.e., AI49-1–6) were tested for their ability to induce an antigen-specific humoral immune response (Ab3) within and across species barriers. This is the classical criterion to define if anti-idiotypic (Ab2) mAb express the internal image of Ab1, thus mimicking a B cell epitope on the antigen. These studies were performed in mice, rats and rabbits to analyze Ab3 humoral immune responses. Mice, in groups of three, were immunized with 25 - 50 μg purified Ab2, AI49-1–6, and a control immunoglobulin (COL-1). Rats, also in groups of three, were immunized with 25 μg AI49-3–5. One rabbit each was administered 50 μg AI49-1–6. Sera were tested in solid-phase radioimmunoassay for reactivity against TAG-72 and an irrelevant control antigen 14 days following each boost. Sera from all rats and three out of six rabbits were also tested for specific reactivity to the Ab2 immunogen. All sera tested displayed strong titers of antibody reactive with the idiotype of the Ab2 immunogen (Fig. 4). On the other hand, none of the antisera derived from the Ab2-immunized mice, rats, and rabbits gave rise to antibody specific for TAG-72 during the 4-month period of biweekly immunizations.

Ab3 antisera derived from immunized rats and rabbits were analyzed for the presence of anti-Ab2-specific immune responses to ensure the animals were responding to immunization. Figure 4 illustrates representative results from one rabbit immunized with mAb AI49-5. The serum was tested for its ability to inhibit the binding of ^{125}I-radiolabeled CC49 to purified AI49-5 coated on the microtiter plate. AI49-5 Ab3 serum derived 14 days following the second immunization (day 28) demonstrated specific inhibition. On the other hand, the AI49-5 rabbit preimmunization serum and an Ab3 rabbit serum against a different Ab2 (AI49-3) did not compete. These data indicated that the rats (immunized with AI49-3–5) and rabbits (immunized with Ab2 AI49-1–6) tested in this manner elicited specific Ab3 immune responses to the Ab2 idiotype that was utilized as immunogen. Titers to the Ab2 idiotype were also shown to rise with subsequent immunizations (data not shown).

Humoral Ab3 responses elicited by anti-idiotypic antibodies to COL-1 (CAI-1–5). Studies were undertaken to determine whether Ab2 mAb CAI-1–5 express the internal image of the COL-1 idiotype (Ab1) thus mimicking an epitope on CEA (Ag). These studies were done in Balb/c and C57BL/6 (three per group) mice and New Zealand rabbits (one or two per group) to analyze the induction of antigen-specific Ab3 humoral immune responses. All animals were immunized as described in Materials and methods with 50 μg purified Ab2 antibodies coupled to KLH. Preimmunization and serum samples taken 14 days following each boost were tested in ELISA against purified CEA and an irrelevant control antigen, thyroglobulin. None of the Ab2 immune sera demonstrated antibody specifically reactive to purified CEA during the 4-month immunization period.

All of the Ab3 rabbit sera were tested for the presence of antibody reactive with the COL-1 idiotype utilized as immunogen. Figure 5 illustrates that the purified Ab3 sera obtained 14 days following the second immunization (day 28) contained antibodies specifically reactive with the Ab2 utilized as immunogen but not with the other COL-1 anti-idiotypic antibodies. Specific anti-Ab2 immune responses were noted in all three rabbits tested. The anti-CAI-1 rabbit Ab3 serum was shown to react with purified CAI-1 Ab2 mAb

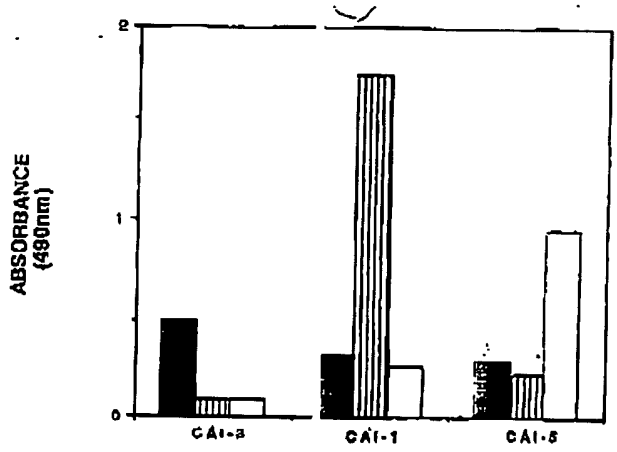

Fig. 5. Reactivity of rabbit Ab3 sera in binding to the COL-1 anti-idiotypic antibodies (Ab2). New Zealand White rabbits were immunized at 2-week intervals with purified COL-1 anti-idiotypic antibodies CAI-1, 3, and 5 coupled to keyhole limpet hemocyanin as described. Rabbit Ab3 sera obtained 14 days after the second immunization were tested for reactivity to their Ab2. Anti-idiotypic antibodies to COL-1 mAb, designated CAI-1, 3 and 5, were coated to microtiter plates (50 ng/well) overnight at 4° C. The rabbit sera against the Ab2 species, CAI-1 (*striped boxes*), CAI-3 (*black boxes*) and CAI-5 (*white boxes*), were diluted in PBS with 1% mouse serum in order to adsorb out anti-(mouse IgG) responses. A 1 : 3125 dilution of each serum was incubated on the antigen plates for 1 h, 37°C. Following a wash step, the plates were treated for 1 h with horseradish-peroxidase-conjugated *Staphylococcus aureus* protein A. The *o*-phenylenediamine chromogen was added for 10 min for the detection of the bound IgG complexe

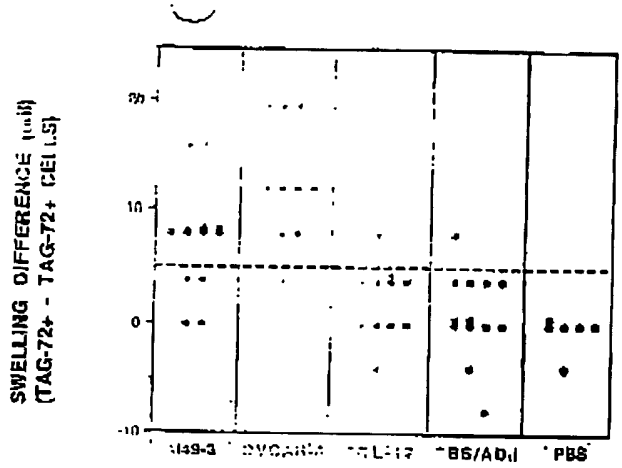

Fig. 6. Anti-idiotypic antibody (Ab2) induction of delayed-type hypersensitivity (DTH) responses to human tumor cells expressing TAG-72 (Ag). Balb/c mice were immunized with 1.5×10^7 X-irradiated hybridoma cells secreting the anti-idiotypic antibody (AI49-3). Hybridoma cells secreting a control IgG (COL-1), PBS in adjuvant or PBS alone were used as control immunogen. Human ovarian carcinoma cells, OVCAR-3, expressing TAG-72 were immunized as a control for a positive DTH response (TAG-72). Seven days following the last boost, each mouse received an injection of 5×10^5 X-irradiated OVCAR-3 into one footpad. As a control for non-specific swelling, the mice received an injection of 5×10^5 X-irradiated TAG-72 negative human embryonic fibroblast cells, MRC-5, in the contralateral footpad. DTH responses were measured 48 h later and expressed in mm (0.0254 mm) as the difference between footpad swelling. The *dotted line* represents an arbitrary baseline for a positive response. Each *dot* represents one mouse. PBS/ADJ, PBS in adjuvant

but not to purified CAI-5 Ab2 mAb. Likewise, anti-CAI-3 rabbit serum only bound to CAI-3 and anti-CAI-5 only bound to CAI-5.

Induction of cell-mediated immunity by Ab2

Ab2 induction of DTH in tumor cells that express TAG-72. Studies were conducted to determine whether immunization of mice with the AI49 anti-idiotypic antibodies can induce cell-mediated immune responses to TAG-72. Two preliminary DTH experiment were performed in which mice were immunized with X-irradiated hybridoma cells secreting AI49-3, 4, or 5 (see Materials and methods). These were three of the Ab2 species that appeared to differ from each other on the basis of the fine binding specificity competition assays. Ab2 AI49-3 was the only antibody that demonstrated differential swelling in response to challenge with the TAG-72-expressing OVCAR-3 ascites cells in 6 out of 8 animals (mean of 203 μm, 8 "mil") (Fig. 6). In addition, purified anti-idiotypic antibodies, AI49-3, 4 and 5 coupled to KLH were tested at one dose level using the same immunization regimen and did not induce DTH responses (data not shown).

A summary of the DTH results from four experiments is shown in Fig. 6. Utilizing 127 μm (5 "mil") as an arbitrary baseline level of a positive DTH response, 9 out of 13 mice immunized with the hybridoma secreting Ab2 AI49-3, demonstrated positive swelling responses (Fig. 6). On the other hand, only 2 out of 32 mice immunized with one of the controls (a hybridoma secreting an isotype-matched control IgG emulsified in IFA, PBS emulsified in IFA or PBS alone) demonstrated responses above the baseline for differential swelling. As a control for a positive DTH response, the irradiated OVCAR-3 cells were also used as immunogen as described above. Out of 11 animals, 10 demonstrated a differential DTH response to a footpad challenge with OVCAR-3 and MRC-5 cells respectively. Thus, AI49-3-Ab2-secreting hybridoma cells were able to elicit significant DTH responses to TAG-72-expressing human tumor cells compared to TAG-72-negative human embryonic fibroblast cell line (Fig. 6).

A two-tailed Student's *t*-test was utilized to analyze the statistical significance of the difference between the means of the observed responses between groups immunized with either the hybridoma cells secreting anti-idiotypic antibody or with the control immunogens described above. These calculations were conducted in such a manner to account for variation between individual experiments. Table 4 reports the *P* values calculated from the combinations of pairwise comparisons of observed responses between the groups of immunogen. AI49-3 significantly induced antigen-specific responses to the TAG-72-expressing ascites cell line, OVCAR-3, when compared to all of the control

Table 4. Statistical analysis of delayed-type hypersensitivity (DTH) responses induced by the CC49 anti-idiotype antibody, AI49-3[a]

Immunogen: pairwise comparisons				
Group 1 (cells)	Group 2 (cells)		(*df*)[b]	*P*
AI49-3	Controls[c]	1.150	(39)	<0.001
AI49-3	COL-12	1.880	(20)	0.010
OVCAR-3	Controls	1.949	(37)	<0.001
OVCAR-3	COL-12	1.872	(18)	0.001
PBS/ADJ[d]	COL-12	0.0200	(20)	1.000

[a] Mice were immunized twice with the immunogens AI49-3 hybridoma cells, TAG-72+ OVCAR-3 cells, control hybridoma cells secreting COL-12, PBS emulsified in adjuvant and PBS alone. Seven days following the last immunization, mice were challenged by administering 5×10^5 OVCAR-3 cells in one footpad and 5×10^5 MRC-5 human fibroblast cells in the other footpad. After 48 h, the footpads were measured (see Materials and methods) and DTH was determined as the difference in footpad swelling. A two-tailed Student's *t*-test of significance was utilized to calculate the *P* values of the differences of the mean DTH responses between groups immunized with the hybridoma cells secreting the anti-idiotypic antibody (AI49-3) versus those that were immunized with either all of the control immunogen (Controls) or versus only those that received the hybridoma cells secreting an irrelevant immunoglobulin (COL-12). Comparisons were also made between the mean DTH responses observed between groups immunized with OVCAR-3 cells versus the mean response observed between groups immunized with the control immunogen or the hybridoma cells secreting the irrelevant immunoglobulin, mAb COL-12. From the differences between the means, *t* was calculated and *P* values were determined [41]

[b] Degrees of freedom

[c] Controls included irradiated hybridoma cells secreting isotype-matched immunoglobulin COL-12, PBS emulsified in Freund's adjuvant, or PBS alone

[d] PBS emulsified in Freund's adjuvant

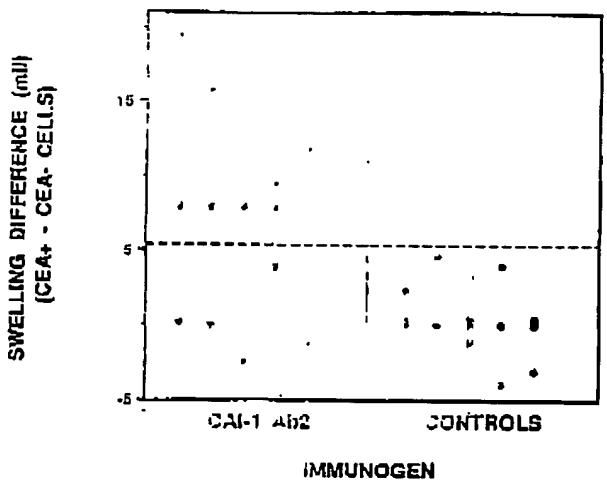

Fig. 7. Anti-idiotypic antibody (Ab2) induction of DTH responses to CEA-transduced murine tumor cells. C57BL/6 mice were immunized as described in the text with X-irradiated hybridoma cells (40 Gy) secreting the anti-idiotypic antibody (Ab2) (CAI-1), or hybridoma cells secreting a control isotype-matched mAb, D612 that is non-reactive with CEA [38]. Seven days following the boost, each mouse was challenged with an injection of 5×10^5 X-irradiated murine tumor cells expressing CEA in one hind footpad. To control for non-specific swelling, 5×10^5 X-irradiated murine non-transduced tumor cells were injected into the opposite footpad. After 48 h, DTH responses were measured in *mil* (0.0254 mm) as the difference between tumor swelling. The *doned line* is an arbitrary value for a positive response. Each *Ab1* represents one mouse

immunogens ($P < 0.001$) or the control hybridoma COL-12 immunogen alone ($P = 0.01$). As a positive immunogen, OVCAR-3 induced significant DTH responses compared to all of the control immunogens combined ($P < 0.001$) and to COL-12 hybridoma alone ($P < 0.001$). In contrast, no differences were observed between groups of mice that received PBS emulsified in IFA compared to responses observed in the groups of mice immunized with the COL-12 hybridoma cells ($P = 1.0$).

Ab2 induction of delayed-type hypersensitivity responses to cells expressing CEA. Studies were done to examine the ability of Ab2 mAb to elicit specific cell-mediated immune responses to CEA on the surface of a tumor cell. Recently we have reported on the generation of a murine C57BL/6 colon adenocarcinoma cell line that has been transduced with the gene for human CEA (MC-38-CEA-2) [44]. The availability of these cells as well as the non-transduced MC-38 cells (CEA-negative) offered an excellent model to test specificity. Figure 7 illustrates the results of a series of three DTH experiments utilizing CAI-1 Ab2 mAb as immunogen. CAI-1 was selected for these experiments because initial experiments demonstrated that CAI-1, out of all of the anti-idiotypic antibodies to COL-1, could elicit DTH responses to the CEA-transduced cells. Data from this experiment aare included in Fig. 7. With an arbitrary baseline for a positive DTH reaction of 140 μm (5.5 "mil"), 8 out of 13 mice immunized with CAI-1 Ab2 mAb showed significant ($P < 0.001$) responses to the CEA-transduced murine tumor cells (average = 190 μm) compared to the mice receiving the control immunogen (average = 6 μm). These data demonstrate that mAb CAI-1, could induce statistically significant cell-mediated immunity directed against tumor cells expressing human CEA (Fig. 7).

Discussion

This paper explores one approach of active specific immunotherapy, the use of monoclonal anti-idiotypic antibodies as surrogate immunogens to induce immune responses to two carcinoma-associated antigens, TAG-72 and CEA. In this report, [illegible] anti-idiotypic antibodies to mAb CC49 and [illegible] immunizing TAG-72 and CEA respectively, were generated and characterized. These anti-idiotypic antibodies were demonstrated by immunoassays (a) to react specifically with the idiotype of the monoclonal antibody (Ab1) used as immunogen and (b) to inhibit the binding interaction between the Ab1 and antigen. Anti-idiotypic antibodies AI49-3 and CAI-1 could

each induce cell-mediated im mun responses specific for tumor cells that express TAG- 72 and CEA, respectively.

Anti-idiotypic antibodies (ffer a unique opportunity to induce specific immune res onses to monoclonal-antibody-defined epitopes known to be tumor-associated, thus eliminating possible cross-rea ctive responses to other epitopes on an antigen that may be found in normal tissues. The CEA-related antigen, no -specific cross-reacting antigen, found on the surface of human granulocytes is one such example. In addition, th e use of anti-idiotypic antibodies could alleviate the p oblems of purifying large quantities of antigen; for exar ple, the currently available source of CEA is liver metasta es of human colon carcinoma tumors, or xenografts gr wn in athymic nude mice. Moreover, we have found that CEA isolated in this manner varies extensively from lot to l t resulting in heterogeneity in content (data not shown). R combinant expression systems that produce large quantities of full-length, completely glycosylated CEA ave not been developed. At this time, only very small amc unts of TAG-72 have been purified from tumor xenografts in a multistep mAb column affinity purification procedure [46]. The gene coding for the protein backbone of the T AG-72 mucin has not been cloned, so no recombinant exp ession system is available.

"Mapping" of epitopes on an antigen by competitive binding analysis with monocl nal antibodies sometimes cannot distinguish between ant bodies that bind to identical epitopes, overlapping sites or t at bind separate sites of the antigen but cross-compete be ause of spatial hindrance. Reciprocal antibody competit on binding analysis of a panel of anti-TAG-72 mAb s ggested that mAb CC49, CC112, CC40, and CC50 rec gnize similar or adjacent epitopes on the TAG-72 molec ule as determined by competition assays [27]. However, ll seven radiolabeled anti-idiotypic antibodies, A149-1- 7, reacted uniquely with mAb CC49 (Ab1) and not with the other CC mAb. These data defined the paratope of CC49 as being distinctive from that of those mAb that re ognize similar epitopes as well as those that bind to diverg nt epitopes of the TAG-72 molecule. Likewise, although t e COL antibodies (COL 1, 4, 6, 7, and 11) were shown to ecognize identical or very similar epitopes of CEA by competition RIA [26], the anti-idiotypic antibodies demor strated that the paratope of COL-1 is distinct from the rest These Ab2 reagents thus recognize private epitopes four d only on mAb CC49 and COL-1, respectively, and can be utilized as unique identifiers of only those idiotypes.

Attempts to generate TAG-7 2 or CEA-specific humoral responses by immunization of odents with the Ab2 were unsuccessful; no antigen-specif c antibody reactivity was observed in either system. On explanation may be that pre-existing Ag+ and Id+ B Cell clones are either absent or present only in very small clone: [24]. Evidence now exists that the concept of internal ima ge conformation for anti-idiotypic antibodies may only apply in special circumstances [24]. Most of the inc uced antigen-specific responses can be explained by th anti-clonotypic stimulation of Id+ B cell clones that are ither primed by disease or those that already existed and v ere committed to producing the antigen-specific immune response. A second possible explanation for this observa ion may be that there is a modulation of Ab3 humoral immune responses against antigen that may have been below the level of detection at the time of serum testing. Finally, these Ab2 may not be entirely paratopic in nature: they may not contain enough contact residues to the paratope of the idiotype to induce an antigen-specific Ab3 antibody response [24]. An explanation for the TAG-72 system might be that it is difficult to generate humoral immunity to a carbohydrate epitope employing the protein of the Ab2 immunoglobulin. However, humoral Ab3 responses have been demonstrated that recognize polysaccharide epitopes of bacterial antigens [51].

At this time, the mechanism for cellular immune recognition of TAG-72 remains unknown. CC49 has been shown to recognize a carbohydrate epitope [27]. Antigen-specific T cell immunity against a carbohydrate moiety induced by an Ab2 is an unexpected observation since it is believed that carbohydrate itself can not induce T cell immunity. On the other hand, full characterization of the molecule may reveal that mAb CC49 (Ab1) recognizes a partially glycosylated epitope. This would allow the anti-idiotype to contain a similar sequence that could be processed and presented by antigen-presenting cells to induce T cell immune responses to TAG-72 on the surface of tumor cells. Cytolytic T cells have been described that recognize a known peptide on a mucin molecule, MUC-2 [1].

Utilizing a DTH assay, several laboratories have reported that anti-idiotypic antibodies could induce cellular immunity in both murine and human tumor systems [29, 42]. In the studies reported here, AI49-3 could induce a DTH response in mice to TAG-72 on the surface of the human ovarian carcinoma ascites cell line, OVCAR-3. In this assay we utilized xenogeneic tumor cells because no syngeneic tumor model exists at this time. Mice do not naturally express TAG-72 on their cells. However, repetition of experiments and the use of many controls as well as statistical analysis confirmed that the DTH responses we observed were not likely to be due to xenogeneic responses.

The beneficial responses induced by anti-idiotypic antibodies have been frequently reported in both experimental systems and in some human clinical investigation. Many anti-idiotypic antibodies have been described that elicit humoral Ab3 responses to tumor-associated antigen in rodent and human antigen systems. This has classically been used as a criterion for proceeding with antitumor effects in model systems or for going on to clinical trials. However, one anti-idiotypic antibody that could not induce Ab3 antibodies to a syngeneic antigen associated with a rat sarcoma could induce an antitumor effect [7]. However, the same anti-idiotypic antibody emulsified in adjuvant could induce Ab3 but could not inhibit tumor growth. Other anti-idiotypic antibodies have previously been demonstrated to elicit DTH responses to tumor antigens [29, 42]. One of these reported Ab2 could inhibit tumor growth in syngeneic rodent model systems [42]. In humans, the development of an antiidiotypic antibody response in patients administered an antitumor-associated antigen mAb to colon carcinoma has been reported to correlate with clinical improvement and long remission from disease [33, 34]. Furthermore, patients administered the same mAb demonstrated specific

DTH responses to the Ab2 that were reported to correlate with complete remission from disease (albeit in a few cases) [35]. Clinical trials with Ab2 are currently ongoing for melanoma [4, 36] and colorectal carcinoma patients [18]. To date, few clinical trials have drawn these types of correlations.

Many anti-idiotypic antibodies have been described to monoclonal antibodies that recognize CEA [3, 8, 13, 30, 37, 51]. The majority of these studies describe Ab2 species that induce Ab3 humoral immune responses specific for CEA [3, 8, 13, 30, 51] characterized by Western blot [13], immunoprecipitation [3] and immunoassays [3, 8, 13, 30, 51], as well as by immunohistochemical staining of colon carcinoma tissue sections [3]. One study has reported an Ab2 to an anti-CEA mAb that can induce a cell-mediated immune response [8]. In this study tumor-infiltrating lymphocytes (TIL) obtained from colon carcinoma patients and stimulated in vitro with an anti-idiotypic mAb were shown to proliferate in response to purified CEA. No proliferation was observed to CEA in those TIL stimulated with a control immunoglobulin. The studies reported here are the first to describe an Ab2 initiating a delayed-type hypersensitivity cellular immune response to CEA-expressing cells. This is also the first report describing anti-idiotypic antibodies to an anti-TAG-72 mAb (CC49); one of these mAb was also shown to mediate a cellular immune response. Cell-mediated immune responses such as DTH have been implicated in playing a role in tumor rejection. Therefore, these anti-idiotypic antibodies may be useful as potential immunogens for active specific immunotherapy protocols of a range of human carcinomas.

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